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Antimicrobial Activity of a Newly Identified Bacteriocin of *Bacillus cereus*

GINO NACLERIO,[1] EZIO RICCA,[1] MARGHERITA SACCO,[2] AND MAURILIO DE FELICE[1]*

Department of General and Environmental Physiology, Section of Microbiology, University Federico II, via Mezzocannone 8, 80134 Naples,[1] and International Institute of Genetics and Biophysics, via G. Marconi 12, 80125 Naples,[2] Italy

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A bacteriocin-producing *Bacillus cereus* strain was isolated. The bacteriocin, here called cerein, was shown to be active specifically against other *B. cereus* strains and inactive against all other bacterial species tested. Cerein was detected in the culture supernatants of stationary-phase cells, and its appearance was inhibited by induction of sporulation. The bactericidal activity of cerein was insensitive to organic solvents and nonproteolytic enzymes, partially stable to heat, and active over a wide range of pH values. Direct detection of antimicrobial activity on sodium dodecyl sulfate-polyacrylamide gel suggested an apparent molecular mass of about 9 kDa.

The gram-positive spore-forming bacterium *Bacillus cereus* is a widely distributed organism in the environment and can be easily isolated from a variety of foods, including dairy products, meats, spices, and cereals. Because of its high incidence in dairy products, *B. cereus* is considered the most important spore-forming species in milk and is responsible for spoilage of pasteurized milk (7, 24). In spite of the initial low number of *B. cereus* cells, this organism quickly becomes the predominant species in pasteurized milk. The total number of *B. cereus* cells decreases when the pH of the medium becomes strongly acidic, probably as a consequence of growth of lactic acid bacteria (18).

B. cereus has been recognized as the agent of two distinct food-borne illnesses, the emetic syndrome, occurring within 1 to 5 h after ingestion of contaminated food and characterized by nausea and vomiting, and the diarrheal syndrome, occurring 8 to 16 h after ingestion and characterized by abdominal cramps, diarrhea, and rectal tenesmus (17). The diarrheal response has been proposed to result from the ingestion of large amounts of *B. cereus*, while the emetic response is likely to result from ingestion of preformed toxin in food (8). At least two distinct enterotoxins have been recognized: a heat-stable one with an apparent molecular mass of 5,000 Da and an unstable one with an apparent molecular mass of about 50,000 Da (22). However, the role of proteases (4, 10, 12, 14) and hemolysins (2, 3) in the pathogenesis is still unclear.

The present report describes the identification of a protein with bactericidal activity in the culture supernatant of a strain of *B. cereus* isolated in food. This bacteriocin, called here cerein, was active against other *B. cereus* strains, from both lab collections and food isolates, while it had no effect on other bacteria tested. A physiological and biochemical characterization of cerein showed that its synthesis is induced at the beginning of the stationary phase and that it has an apparent molecular mass of about 9,000 Da and is stable after various chemical and physical treatments. The features of cerein, together with its specificity against *B. cereus* strains, make this bacteriocin potentially interesting as an antimicrobial agent for the control of *B. cereus* in foods, without interference with the growth of other bacteria.

* Corresponding author. Electronic mail address: sacco@mvx36.csata.it.

MATERIALS AND METHODS

Strains and media. A strain of *B. cereus* isolated in food identified in our laboratory, was designated GN105 and cultured in brain heart infusion (BHI) broth (Difco, Detroit, Mich.). The sporulation medium utilized was Difco Sporulation Medium (DSM) prepared as described by Nicholson and Setlow (19). The isolate was characterized as a *B. cereus* strain by agar base tests (Biolife, Milan, Italy) according to Holbrook and Anderson (11). *B. cereus* 6A2 (obtained from the *Bacillus* Genetic Stock Center, Ohio State University) was used as an indicator strain in all the antimicrobial assays, except where otherwise indicated.

Chemicals. All the chemicals were purchased from J. T. Baker, (Deventer, Holland) and from Sigma (St. Louis, Mo.).

Cerein preparation. Culture supernatants of strain GN105, grown in glucose-supplemented BHI broth at 30°C, were collected 2 h after the beginning of the stationary phase. The samples were centrifuged, filter sterilized (0.45 μm-pore-size filter), and precipitated with 80% ammonium sulfate. After removal of ammonium sulfate by Centricon 3 dialysis (3,000, molecular weight cutoff) (Amicon, Beverly, Mass.), the total protein concentration, determined according to the method of Lowry et al. (16), was about 1.5 mg/ml.

Antimicrobial assay. Tryptone-yeast extract (TY) agar (0.7%), containing 10^7 cells of the indicator strain per ml, was overlaid on 1.5% TY agar plates. When the soft agar was hardened, 5 μl of the cerein preparation was spotted on the plate, and after about 16 h of incubation at 30°C, an inhibition halo was clearly visible.

Effects of enzymes, organic solvents, heat, and pH on bacteriocin. Enzymes (100 μg/ml) and 10% organic solvents (see Table 3) were added to 150-μl samples of culture supernatants. Enzyme-treated samples were incubated 1 h at 37°C (42°C in the case of proteinase K), and solvent-treated samples were incubated for 1 and 5 h at 25°C, before being tested for antimicrobial activity. The effects of heat stability and pH on cerein were analyzed by assaying the bactericidal activity after 15 min of incubation at 45, 60, 75, and 90°C and

TABLE 1. Antimicrobial spectrum of cerein

Organism	No. of strains inhibited/no. tested
Bacillus cereus	11/11
Bacillus subtilis	0/5
Escherichia coli	0/4
Listeria innocua	0/1
Listeria monocytogenes	0/1
Shigella sp.	0/1
Streptococcus cremoris	0/1
Streptococcus diacetolactis	0/1
Streptococcus lactis	0/6
Staphylococcus aureus	0/1

TABLE 2. Cerein production and/or secretion in various growth conditions

Medium[a]	Temp (°C)	Cerein activity[b]
TY	30	0
TSS	30	0
DSM	30	0
BHI	30	10.0
BHI	25	0
BHI	37	5.0

[a] Tris Spizizen salt (TSS) minimal medium was supplemented with 0.4% glucose and 0.1% Casamino Acids (19); BHI broth was supplemented with 0.1% glucose.

[b] Cerein activity is reported as the diameter (in millimeters) of the inhibition halo in plate assays (see Materials and Methods).

after 1- and 5-h incubations at 30°C in 50 mM phosphate buffer (pH 6.0), adjusted to the various pH with HCl and NaOH.

Mode of action. An exponentially growing culture of the indicator strain *B. cereus* 6A2 (10^6 cells) was suspended in 50 mM phosphate buffer (pH 6.0) and exposed for a maximum of 180 min to various concentrations of the bacteriocin. At various times the number of surviving bacteria (CFU per milliliter) was determined by plate counting.

Direct detection of bacteriocin activity on gel. Proteins contained in two 60-μl samples of culture supernatants of strain GN105 were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) (50 mA). After 5 h, the gel was removed and cut into two vertical parts. One part of the gel, containing one sample and the molecular weight standards, was stained with Coomassie brilliant blue R250 (Sigma). The other part, containing the other sample, was tested for antimicrobial activity by the method of Bhunia et al. (5), with the following modifications. The gel was fixed immediately by a 3-h treatment in 20% isopropanol–10 mM Tris-HCl (pH 7.5) and washed for 1.5 h in 10 mM Tris-HCl (pH 7.5). The gel was then placed into a petri dish and overlaid with 7 ml of 0.7% agar containing 10^6 cells of the indicator strain. The dish was then incubated at 30°C for about 16 h and analyzed for an inhibition halo.

RESULTS

A protein secreted by *B. cereus* shows antimicrobial activity. Culture supernatants of several strains of *B. cereus* isolated in food were filter sterilized and tested for the presence of antimicrobial activities by halo inhibition assay on agar plates. Strain GN105 was found to produce and secrete a protein factor, called cerein hereafter, which strongly reduced the growth of the *B. cereus* indicator strain utilized. Cerein action appeared to be highly specific against other *B. cereus* strains, whereas it was not active against various gram-positive and gram-negative species tested (Table 1).

Cerein synthesis and/or secretion was observed when strain GN105 was grown in glucose-supplemented BHI broth at 30°C, while no antimicrobial activity was observed for growth in various other minimal or rich media (Table 2). Induction of sporulation also inhibited the appearance of cerein, as observed when strain GN105 was grown in DSM medium (Table 2). Finally, no bacteriocin was detected when strain GN105 was grown in BHI broth at 25°C, while only a reduced amount of cerein was found at 37°C (Table 2). Cerein synthesis and/or secretion was also dependent on the growth phase of the *B. cereus* strain. Culture supernatants of strain GN105, grown in glucose-supplemented BHI broth at 30°C, were collected at various times during exponential and stationary growth. Samples were filter sterilized and tested for bacteriocin activity by halo inhibition assays on agar plates. As shown in Fig. 1, bacteriocin activity was not detected in samples collected during exponential growth but was detected in samples collected at the beginning of the stationary phase. A decrease of the bactericidal activity of culture supernatants occurred about 3 h after the beginning of the stationary phase (Fig. 1).

Effects of enzymes, organic solvents, heat, and pH on cerein activity. A cerein preparation obtained after ammonium sulfate precipitation was tested for sensitivity (measured as the loss of inhibition in an agar plate assay) to various enzymes, organic solvents, heat, and pH values. As shown in Table 3, the antimicrobial activity was not affected by treatment with lysozyme, ribonuclease A, DNase, or any of the organic solvents used, while it was completely lost after treatments with trypsin, chymotrypsin, and protease K (Table 3), thus suggesting the proteinaceous nature of the bacteriocin. Cerein was also partially stable to heat treatments; the activity was maintained during treatments up to 75°C and disappeared only after 15 min of incubation at 90°C (Table 3). Cerein was also active over a wide pH range, with only partial sensitivity to extremely acidic or extremely basic conditions (Table 4).

Mode of action. To determine whether cerein had a bactericidal or a bacteriostatic effect, various concentrations of

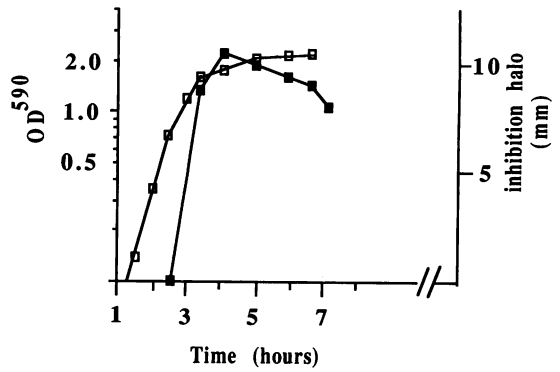

FIG. 1. Correlation between growth and cerein appearance in the medium. Open symbols refer to the growth curve of strain GN105 in glucose-supplemented BHI broth at 30°C. Closed symbols indicate the diameter of the inhibition halo observed in agar plate assays with samples collected at various times during growth. OD_{590}, optical density at 590 nm.

TABLE 3. Effects of enzymes, organic solvents, and heat on cerein activity

Treatment[a]	Activity[b]
None	10.0
Trypsin	0
Chymotrypsin	0
Protease K	0
DNase	10.0
Ribonuclease A	10.0
Lysozyme	10.0
Acetone[c]	10.0
Acetonitrile[c]	10.0
Ethyl alcohol[c]	10.0
Chloroform	10.0
Methyl alcohol[c]	10.0
Toluene[c]	10.0
Incubation (15 min) at:	
45°C	10.0
60°C	10.0
75°C	7.5
90°C	0

[a] The enzyme concentration was 100 μg/ml; the total protein concentration of the crude cerein preparation was 1.5 mg/ml.
[b] Cerein activity is expressed as the diameter (in millimeters) of the inhibition halo in plate assays (see Materials and Methods).
[c] A 10% (vol/vol) concentration was used.

the bacteriocin were added to the indicator *B. cereus* strain suspended in phosphate buffer (pH 6.0). The number of surviving indicator bacterial cells was determined by plate counting at various times after the addition of the bacteriocin. As shown in Fig. 2, the incubation of cells with cerein reduced the number of surviving cells, thus suggesting its bactericidal activity. The effect increased with the increase in the protein concentration and the incubation time.

Cerein is a 9-kDa protein. SDS-PAGE (Fig. 3A) revealed that stationary-phase culture supernatants of strain GN105 contained about a dozen proteins. The 15% polyacrylamide gel, containing stationary-phase culture supernatants after ammonium sulfate precipitation, was cut into two vertical parts. The part of the gel containing the sample and the molecular weight markers was stained, while the remaining part, containing only the sample, was fixed and used for direct detection of antimicrobial activity by the method of

TABLE 4. Effect of pH on cerein activity

pH	Activity[a] after	
	1 h	5 h
1	6.0	6.0
2	7.5	7.5
3	10.0	10.0
4	10.0	10.0
5	10.0	10.0
6	10.0	10.0
7	10.0	10.0
8	10.0	10.0
9	10.0	10.0
10	10.0	10.0
11	10.0	10.0
12	10.0	10.0
13	4.5	4.5

[a] Cerein activity is indicated as the diameter (in millimeters) of the inhibition halo in plate assays at 30°C (see Materials and Methods). The total protein concentration of the crude cerein preparation was 1.5 mg/ml.

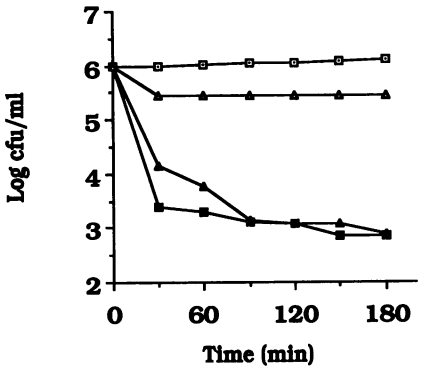

FIG. 2. Effects of various concentrations of cerein on the growth of the indicator strain. Exponentially growing cells of the indicator strain were incubated with the bacteriocin at 30°C, and samples were tested for growth after various incubation times. The protein concentrations of the cerein preparations were 0 (□), 15 μg/ml (▲), 37.5 μg/ml (▲), and 75 μg/ml (■). For other details, see Materials and Methods.

Bhunia et al. (5). As shown in Fig. 3B, the bactericidal activity of cerein is associated with a band having an apparent molecular mass of about 9 kDa.

DISCUSSION

In the last few years a variety of antagonistic factors, including metabolic end-products, antibiotic-like compounds, and bacteriocins, mostly produced by lactic acid bacteria, have attracted attention for their potential use as food additives (15). Most of the bacteriocins show a narrow spectrum of action, as they inhibit strains closely related to the producer organisms, while only few bacteriocins inhibit diverse groups of gram-positive bacteria (13). We report here the identification and a partial characterization of a bacteriocin produced by a strain of *B. cereus* isolated in food. This

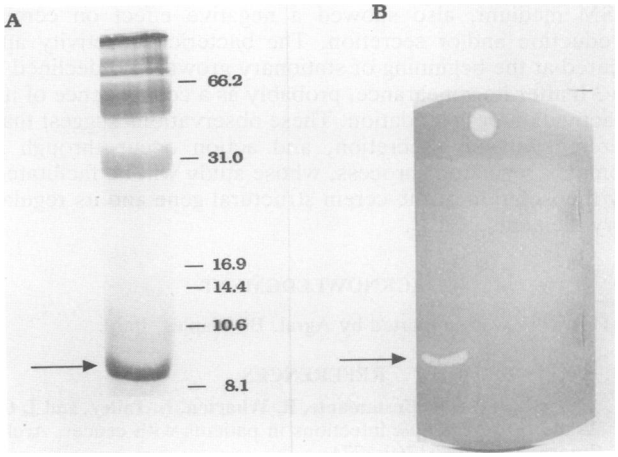

FIG. 3. SDS-PAGE of stationary-phase culture supernatants of *B. cereus* GN105. (A) Stained portion of the gel. The arrow indicates the region of the gel responsible for the inhibition halo shown in panel B. (B) Portion of the gel overlaid with the indicator strain as described in Materials and Methods. The arrow indicates the inhibition halo observed after overnight incubation at 30°C. The inhibition halo in the upper part of panel B was obtained with an aliquot of the same sample used for electrophoresis.

bacteriocin, which we call cerein, differs from other bacteriocins so far identified in that it does not inhibit lactic acid bacteria and is active exclusively against other *B. cereus* strains. This specificity of cerein could be useful for depressing *B. cereus* growth without interfering with other bacteria normally present in food preparations.

B. cereus is considered an emerging food-borne pathogen and is becoming increasingly of concern as a potential determinant of food poisoning, because of the occurrence of enterotoxic strains able to grow at temperatures below 8°C (20, 23). Recently, *B. cereus* has been recognized as the agent of two distinct food-borne illnesses, the emetic syndrome and the diarrheal syndrome (21). Bacteremia, meningitis, pneumonia, endocarditis, and wound and ocular infections have been also reported as consequences of *B. cereus* infections in patients with cancer and acute leukemia (1, 9).

Cerein showed a bactericidal mode of action. As shown in Fig. 2, a decrease in CFU per milliliter was observed after exposure of the indicator strain to various concentrations of the bacteriocin. Although our experimental approach did not allow verification of whether cell lysis occurred, these results suggested that cerein acts as a bactericidal agent. The proteinaceous nature of the bacteriocin was indicated by the loss of cerein activity after treatment with proteolytic enzymes, and the apparent molecular mass of the bacteriocin was estimated to be about 9 kDa by direct detection of bactericidal activity after SDS-PAGE (Fig. 3). As found earlier with other bacteriocins produced by mesophilic bacteria (6, 21), cerein activity was not affected by treatments with several organic solvents. The stability of cerein over a wide pH range and after heat treatments up to 75°C indicated that its bactericidal function is active in a variety of different conditions; this is an interesting feature in view of its potential use as a food additive.

Synthesis and/or secretion of the bacteriocin appeared to be strictly regulated. Cerein was detected only in the culture supernatants of cells grown in glucose-supplemented BHI broth at the optimal temperature of 30°C. A temperature shift to 25 or 37°C resulted in complete disappearance of or a decrease in the bactericidal activity, respectively. Induction of sporulation, obtained by growing *B. cereus* GN105 in DSM medium, also showed a negative effect on cerein production and/or secretion. The bactericidal activity appeared at the beginning of stationary growth and declined 2 to 3 h after its appearance, probably as a consequence of its inactivation or degradation. These observations suggest that cerein synthesis, secretion, and action occur through a complex regulatory process, whose study will be facilitated by the isolation of the cerein structural gene and its regulatory elements.

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REFERENCES

1. **Banerjee, C., C. I. Bustamante, R. Wharton, E. Talley, and J. C. Wade.** 1988. *Bacillus* infections in patients with cancer. Arch. Intern. Med. **148:**1769–1774.
2. **Beecher, D. J., and J. D. MacMillan.** 1990. A novel bicomponent hemolysin from *Bacillus cereus*. Infect. Immun. **58:**2220–2227.
3. **Beecher, D. J., and J. D. MacMillan.** 1991. Characterization of the components of hemolysin BL from *Bacillus cereus*. Infect. Immun. **59:**1778–1784.
4. **Bernheinmer, A. W., and P. Grushoff.** 1967. Cereolysin: production, purification and partial characterization. J. Gen. Microbiol. **46:**143–150.
5. **Bhunia, A. K., M. C. Johnson, and B. Ray.** 1987. Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl-polyacrylamide gel electrophoresis. J. Indust. Microbiol. **2:**319–322.
6. **Bhunia, A. K., M. C. Johnson, and B. Ray.** 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. **65:**261–268.
7. **Christiansson, A., A. Satyanarayan Naidu, I. Nilsson, T. Wadstrom, and H.-E. Petterson.** 1989. Toxin production by *Bacillus cereus* dairy isolates in milk at low temperatures. Appl. Environ. Microbiol. **55:**2595–2600.
8. **Doyle, M. P.** 1988. *Bacillus cereus*. Food Technol. **April:**181–200.
9. **Funada, H., C. Uotani, T. Machi, T. Matsuda, and A. Nonomura.** 1987. *Bacillus cereus* bacteremia in an adult with acute leukemia. Jpn. J. Clin. Oncol. **18:**69–74.
10. **Gilmore, M. S., A. L. Cruz-Rodz, M. Leimeister-Wachter, J. Kreft, and W. Goebel.** 1989. A *Bacillus cereus* cytolytic determinant, Cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. J. Bacteriol. **171:**744–753.
11. **Holbrook, R., and J. M. Anderson.** 1980. An improved selective and diagnostic medium for the isolation and enumeration of *Bacillus cereus* in foods. Can. J. Microbiol. **26:**753–759.
12. **Johnson, C. E., and P. F. Bonventre.** 1967. Lethal toxin of *Bacillus cereus*. J. Bacteriol. **94:**306–316.
13. **Klaenhammer, T. R.** 1988. Bacteriocins of lactic acid bacteria. Biochimie **70:**337–349.
14. **Kreft, J., H. Berger, M. Härtlein, B. Müller, G. Weidinger, and W. Goebel.** 1983. Cloning and expression in *Escherichia coli* and *Bacillus subtilis* of the hemolysin (cereolysin) determinant from *Bacillus cereus*. J. Bacteriol. **155:**681–689.
15. **Lindgren, S. E., and W. J. Dobrogosz.** 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentation. FEMS Microbiol. Rev. **7:**149–163.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193:**265–275.
17. **Mortimer, P. R., and G. McCann.** 1974. Food-poisoning episodes associated with *Bacillus cereus* in fried rice. Lancet **i:**1043–1045.
18. **Naclerio, G.** Unpublished data.
19. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination and outgrowth, p. 391–450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd, Chichester, United Kingdom.
20. **Schofield, G. M.** 1992. Emerging food-borne pathogens and their significance in chilled foods. J. Appl. Bacteriol. **72:**267–273.
21. **Stoffels, G., J. Nissen-Meyer, A. Gudmunsdottir, K. Sletten, H. Holo, and I. F. Nes.** 1992. Purification and characterization of a new bacteriocin isolated from a Carnobacterium sp. Appl. Environ. Microbiol. **58:**1417–1422.
22. **Thompson, N. E., M. J. Ketterhagen, M. S. Bergdoll, and E. J. Schantz.** 1984. Isolation and some properties of an enterotoxin produced by *Bacillus cereus*. Infect. Immun. **43:**887–894.
23. **van Netten, P., A. van de Moosdijk, P. van Hoensel, D. A. A. Mossel, and I. Perales.** 1990. Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. J. Appl. Bacteriol. **69:**73–79.
24. **Wong, H.-C., M.-H. Chang, and J.-Y. Fan.** 1988. Incidence and characterization of *Bacillus cereus* isolates contaminating dairy products. Appl. Environ. Microbiol. **54:**699–702.